Characterization of Glucose Transport System in Keratinocytes: Insulin and IGF-1 Differentially Affect Specific Transporters

Shlomzion Shen, Efrat Wertheimer,* Sanford R. Sampson, and Tamar Tennenbaum

Faculty of Life Sciences, Gonda-Goldschmeid Center, Bar-Ilan University, Ramat-Gan, Israel; *Department of Pathology, Sackler School of Medicine, Tel Aviv University, Ramat-Aviv, Tel Aviv, Israel

Skin is one of the major tissues displaying chronic diabetic complications. We have studied glucose transport following stimulation with insulin and IGF-1 in cultured mouse keratinocytes. In proliferating cells, acute stimulation with insulin and IGF-1 increased glucose uptake. Insulin translocated glucose transporters 1 and 5, whereas IGF-1 translocated glucose transporters 2 and 3. With differentiation, glucose transporter 3 expression increased and the expression of glucose transporters 1, 2, and 5 decreased. No increase in glucose uptake was observed, however, following stimulation with either hormone. These results indicate that insulin and IGF-1 differentially regulate glucose uptake as well as expression and translocation of specific transporters in skin keratinocytes. ***Key words: glucose transport/glucose transporters/growth factors/insulin/keratinocytes/skin.***

J Invest Dermatol 115:949–954, 2000

Skin is one of the major tissues that display pathologic changes associated with diabetes mellitus (Jelinek, 1993; Sibbald *et al*, 1996). These include dermatologic disorders associated with diabetes such as impaired wound healing, diabetes dermopathy, and other conditions such as necrobiosis lipoidica diabeticorum, waxy skin, and acanthosis nigricans (Huntley, 1993). Although numerous studies on experimental diabetic models have revealed abnormal glucose transport in classical insulin-responsive tissues such as muscle, fat, and liver, detailed studies on other tissues such as skin have not been reported.

Insulin and IGF-1 have each been shown to be important for skin function (Benoliel *et al*, 1997; Smola *et al*, 1998). Both hormones promote proliferation and migration of human and mouse keratinocytes. In addition, insulin is an essential component in the growth medium of human keratinocytes *in vitro* (Tsao *et al*, 1982). Furthermore, a defect in skin development was observed in IGF receptor (IGFR) null mice (Liu *et al*, 1993). As skin is not considered to be a major insulin-responsive tissue, however, little is known about the effects of the insulin family of proteins on regulation of metabolic processes in skin keratinocytes. As is well known, insulin and IGF-1 control glucose transport in numerous tissues. Indeed, defects in glucose transport have been shown to contribute to the development of diabetic complications in various tissues (Huntley, 1993; Jelinek, 1993; Smola *et al*, 1998). The fact that skin dermatologic complications occur in diabetes would appear to indicate that defects in insulin signaling or regulation of glucose metabolism may be involved. Nonetheless, effects of insulin and IGF-1 on glucose transport in skin and its association with growth and differentiation have not been reported.

Manuscript received May 9, 1999; revised August 22, 2000; accepted for publication August 28, 2000.

Reprint requests to: Dr. S. R. Sampson, Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel. Email: sampsos@mail.biu.ac.il

Abbreviation: GLUT, glucose transporter.

The transport of glucose across the cell plasma membrane occurs by facilitative diffusion mediated by a family of transmembrane glycoproteins termed glucose transporters (GLUTs) (Gould and Holman, 1993; Olson and Pessin, 1996). It is currently believed that GLUTs have some degree of tissue specificity. GLUT 1 is known to be ubiquitously expressed (Wertheimer *et al*, 1991), whereas GLUT 2 is mostly expressed in liver, kidney, intestine, and pancreatic β-cells, GLUT 3 is mostly expressed in muscle, heart, and adipocytes, and GLUT 4 is mostly expressed in cardiac muscles, skeleton muscles, and fat cells. GLUT 5 has been identified in small intestine, brain, muscle, and adipose tissue (Takata, 1996). A number of tissues express more than one glucose transporter: skeletal muscle expresses GLUTs 1, 3, and 4, adipose tissue expresses GLUTs 1 and 4, and heart expresses GLUTs 3 and 4 (Gould and Holman, 1993; Pascoe *et al*, 1996). Stimulation of glucose uptake, especially by insulin given acutely, occurs via translocation of the GLUTs from the cytoplasm to the plasma membrane (Klip *et al*, 1993; Cushman *et al*, 1998). Studies on human keratinocytes have shown that these cells seem to express only GLUT 1 (Gherzi *et al*, 1992; Voldstedlund and Dabelsteen, 1997). A detailed analysis of GLUT expression in the various stages of skin differentiation has not been reported, however. Furthermore, although it was found that insulin and IGF-1 increase glucose uptake and GLUT 1 expression (Gherzi *et al*, 1992), detailed studies on GLUT translocation were not done.

The purpose of this study was to characterize the glucose transport system in skin keratinocytes and to examine the effects of insulin and IGF-1 on glucose transport in these cells. In order to do so, we utilized a culture system of mouse keratinocytes. The differentiation state of these cells can be regulated by adjusting the extracellular calcium concentrations (Hennings *et al*, 1980). Primary mouse keratinocytes cultured *in vitro* in medium containing a low concentration of Ca^{2+} (0.05 mM) retain a proliferative basal cell phenotype with a high growth rate. Elevation of calcium concentrations above 0.1 mM Ca^{2+} induces a rapid sequence of events leading to loss of proliferation potential and to differentiation

of keratinocytes. Further elevation of the Ca^{2+} concentration to 1.0 mM is associated with terminal differentiation, formation of cornified envelopes, and cell death. Differentiated cells ultimately slough from the culture dish as mature squames. This program of calcium-induced differentiation *in vitro* closely follows the maturation pattern of epidermis *in vivo* (Yuspa *et al*, 1989).

This study characterizes the glucose transport system in keratinocytes including the expression of several GLUTs. Moreover, we show that insulin and IGF-1 specifically increase glucose transport in skin keratinocytes, and induce distinct changes in expression and translocation of different GLUTs. Preliminary results have been reported.[1]

MATERIALS AND METHODS

Materials Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence was performed with a kit purchased from BioRad (Israel). Polyclonal antibodies to GLUTs 1, 2, 3, and 5 were a gift from Dr. S. Cushman (Diabetes Branch, NIDDK, NIH); horseradish peroxidase antirabbit and antimouse IgG were obtained from Bio-Rad. Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, orthovanadate, and pepstatin were purchased from Sigma (St. Louis, MO). Insulin (HumulinR-recombinant human insulin) was purchased from Lilly France SA (Fergersheim, France). IGF-1 was a gift from Cytolab (Israel).

Isolation and culture of murine keratinocytes Primary keratinocytes were isolated as described previously (Dlugosz *et al*, 1995) from newborn BALB/C mice. Keratinocytes were cultured in Eagle's minimal essential medium containing fetal bovine serum treated with 8% Chelex (Chelex-100, BioRad). To maintain a proliferative basal cell phenotype, the final Ca^{2+} concentration was adjusted to 0.05 mM. Experiments were performed 5–7 d after plating.

Preparation of cell extracts and western blot analysis For crude membrane fractions, lysates were prepared by scraping cells with a rubber policeman into phosphate-buffered saline (PBS) containing 10 μg per ml aprotinin, 10 μg per ml leupeptin, 2 μg per ml pepstatin, 1 mM PMSF, 10 mM ethylenediamine tetraacetic acid, 200 μM $NaVO_4$, and 10 mM NaF. After four cycles of freeze-thaw, cells were homogenized and centrifuged at 4°C for 20 min at 2300 × *g*. The supernatant containing the soluble protein fraction (cytoplasmic extract) was transferred to another tube. This fraction contained both the cytoplasmic proteins and internal membranes where GLUTs are localized prior to translocation. The pellet was resuspended in 250 μl PBS containing 1% Triton X-100 with protease and phosphatase inhibitors. Cells were incubated for 30 min at 4°C and centrifuged at 2300 × *g* at 4°C. The supernatant containing the membrane fraction was transferred to a fresh tube. The cytoplasmic and membrane extracts were frozen at −70°C until used. The purity of the membrane preparations was confirmed by the expression of specific membrane markers (Na/K pump α1 isoform). The purity of the cytoplasmic extract was confirmed by expression of MAPK and p-MAPK, which in our model system of keratinocyte expression is localized in the cytoplasmic extract. The remaining pellet contained cytoskeletal proteins. Protein concentrations were measured using a modified Lowry assay (Bio-Rad DC Protein Assay Kit). According to the protein levels it was routinely confirmed that the relative protein ratio between membrane and cytoplasmic extracts was maintained at 1:7. Western blot analysis of cellular protein fractions was carried out as described previously (Tennenbaum *et al*, 1996).

Glucose transport Glucose transport was evaluated by measuring 2-deoxy-[^{3}H]-glucose according to the method described previously (Wertheimer *et al*, 1990). Briefly, on the day of the experiment, cells were washed three times with PBS. Then, 0.1 mM 2-deoxy-glucose/PBS with tracer amounts of 2-deoxy-[^{3}H]-glucose (1 μCi per plate; ARC, St. Louis, MO) was added to the cells. Uptake was continued for 10 min at 37°C. The reaction was stopped by three quick washes with 1.0 ml cold PBS on ice, and cells were lyzed in 1% Triton X-100. The samples were counted in the [^{3}H] window of a Tricarb scintillation counter. Uptake was linear under these conditions for up to 15 min (**Fig 1**). Mean values were determined from measurements of triplicate samples under each experimental condition for each experiment.

RESULTS

Basal glucose transport We first examined basal glucose uptake in proliferating and differentiating keratinocytes. As mentioned, keratinocytes maintained in a low Ca^{2+} concentration (0.05 mM) express properties of basal proliferating cells. Elevating the Ca^{2+} concentration to above 0.1 mM induces rapid differentiation; cells express specific differentiation markers and cease to proliferate. We measured basal glucose uptake of cells maintained in different concentrations at various time points, and the results are illustrated in **Fig 1**. It can be seen that in both proliferating and differentiating keratinocytes a linear basal uptake was observed in the range of 0–40 min uptake. Maximal glucose uptake was observed in keratinocytes cultured in low Ca^{2+} concentrations. These cells present basal-like keratinocytes that are highly proliferative and metabolically active.

As GLUTs mediate the transport of glucose into cells, we next identified the GLUTs expressed in keratinocytes. As seen in **Fig 2**, keratinocytes at all Ca^{2+} concentrations express GLUTs 1, 2, 3, and 5, but there were changes in GLUT expression as a function of Ca^{2+} concentration. Thus, Ca^{2+}-induced differentiation was associated with increased expression of GLUT 3 and decreased expression of GLUTs 1, 2, and 5.

Effects of insulin and IGF-1 on glucose uptake One of the major functions of insulin is to promote the transport of glucose into cells. As we have found that several GLUTs are expressed in keratinocytes maintained in different Ca^{2+} concentrations, we investigated the effects of insulin and IGF-1 on glucose uptake in these cells. The results are summarized in **Fig 3**. In proliferating keratinocytes maintained in 0.05 mM Ca^{2+}, insulin treatment for 1 h and 24 h increased glucose uptake by 60% and 120%, respectively. In contrast, stimulation with IGF-1 increased glucose uptake by 150% as early as 5 min after addition of the hormone, and by nearly 300% after 15 min (**Fig 3*A***). Similar to stimulation with insulin, glucose uptake by IGF-1 remained elevated (120% above control) for up to 24 h. Thus, the effect of IGF-1 on glucose uptake occurred sooner and was stronger than that of insulin. Surprisingly, although differentiating keratinocytes express several GLUTs, elevation of extracellular Ca^{2+} was

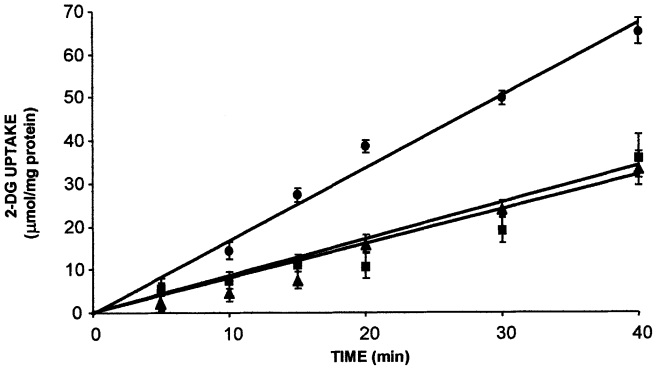

Figure 1. Basal glucose uptake in proliferating and differentiating keratinocytes. Primary keratinocytes were isolated and plated as described in *Materials and Methods*. Proliferating keratinocytes were maintained for 5 d in low Ca^{2+} medium (0.05 mM) until they reached 80% confluency. Differentiation was then induced by elevating the Ca^{2+} concentration in the growth medium from 0.05 mM (●) to 0.12 mM (▲) and 1.0 mM (■). Cells were allowed to differentiate for 24 h. Glucose uptake was carried out as described in *Materials and Methods* for the times indicated. Each symbol represents the mean ± SE of three determinations in five experiments done on separate cultures.

[1]Shen S, Wertheimer E, Bak A, Tennenbaum T, Sampson SR: Proceedings of the International Symposium on Insulin Receptor and Insulin Action, p. 211, 1998 (abstr.)

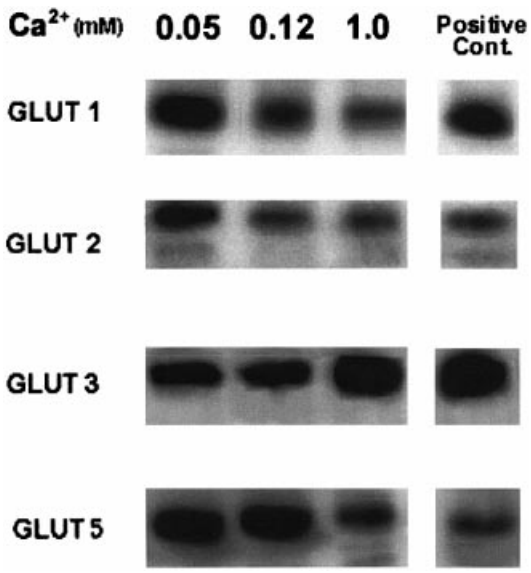

Figure 2. Expression of GLUTs in proliferating and differentiating keratinocytes. Cells were cultivated as described in **Fig 1**. Total cell lysates (20 μg protein) were analyzed by western blotting using specific polyclonal antibodies to each transporter. Brain extract was used as positive control for GLUTs 1 and 3. Kidney extract was used as positive control for GLUT 2 and muscle was used as positive control for GLUT 5. The blots shown are representative of three different experiments.

associated with abolition of the response to both insulin and IGF-1 (**Fig 3*B***). As neither insulin nor IGF-1 had a detectable effect on glucose uptake in differentiating keratinocytes, all subsequent studies were done on basal proliferating keratinocytes.

Effects of insulin and IGF-1 on GLUTs

Acute effects The finding that insulin and IGF-1 increase glucose uptake in proliferating basal keratinocytes suggests that these hormones might differentially activate various GLUTs in these cells. Accordingly, we determined which transporters are affected by insulin and IGF-1. The results are shown in **Fig 4**. As activation of GLUTs is usually associated with translocation of the transporters to the plasma membrane, we initially followed the distribution of GLUTs following acute stimulation with insulin and IGF-1 in keratinocytes. As a plasma membrane marker we used the Na/K pump α1 isoform. As expected (**Fig 4*A***), expression of the Na/K pump α1 isoform was limited to the plasma membrane fraction. It can be seen that insulin translocated both GLUTs 1 and 5 from the cytoplasm to the plasma membrane within 5 min (**Fig 4*A, C***), and this effect was maintained for at least 60 min. Insulin had no effect on any of the other isoforms within this time period. In contrast, IGF-1 translocated both GLUT 2 and GLUT 3 from the cytoplasm to the plasma membrane within 5 min, and these transporters remained elevated in the plasma membrane for at least 60 min. IGF-1 had no effect on any of the other isoforms within this time period (**Fig 4*B, C***). At the same time, administration of insulin or IGF-1 for 60 min did not affect the total expression of the various GLUTs (**Fig 4*D***). Thus insulin and IGF-1 each translocated distinct, separate GLUT isoforms to produce its effects on glucose transport.

Chronic effects One possible explanation for the increased glucose transport with chronic treatment is a change in expression and distribution of the individual transporters from that in nonstimulated cells. To identify which of the GLUTs were affected by chronic insulin or IGF-1 stimulation, we performed western blot analysis of cytoplasmic and plasma membrane extracts from lysates of cells treated with insulin and IGF-1 for 24 h. The results are shown in **Fig 5**(***A***). Following 24 h treatment with insulin, the expression of GLUTs 2 and 3, which were not translocated by

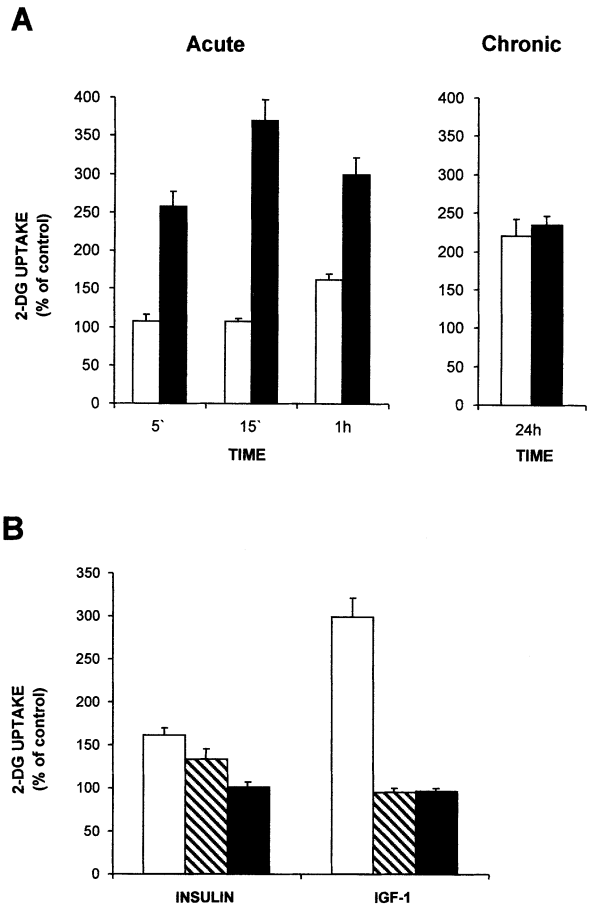

Figure 3. Effects of insulin or IGF-1 on glucose uptake by keratinocytes maintained in different Ca^{2+} concentrations. 2-deoxy-[^{3}H]-glucose uptake was measured as described in *Materials and Methods*. Each bar represents the mean ± SE of three determinations in five experiments done on separate cultures. Values are expressed as a percentage of control, unstimulated cells from the same culture in each experiment. The control level of unstimulated cells was 30 μmol 2-deoxy-[^{3}H]-glucose per mg protein per min. (*A*) Five-day-old keratinocytes were maintained in 0.05 mM Ca^{2+} and stimulated with 10^{-7} M insulin (*open bars*) or 10^{-8} M IGF-1 (*filled bars*) for the designated times. (*B*) Keratinocytes were induced to differentiate by elevation of [Ca^{2+}] from 0.05 mM (*open bars*) to 0.12 mM (*slashed bars*) and to 1.0 mM (*filled bars*) for 24 h. Cells in all Ca^{2+} concentrations were treated for 60 min with insulin (10^{-7} M) or IGF-1 (10^{-8} M).

acute insulin stimulation, was found to be upregulated in both cytoplasmic and plasma membrane extracts. GLUTs 1 and 5, which were translocated by insulin acutely, remained elevated in the plasma membrane for 24 h. In contrast, IGF-1 treatment for 24 h resulted in increased levels of GLUTs 1 and 5 in both cytoplasmic and plasma membrane extracts; these transporters were not affected by IGF-1 given acutely. GLUTs 2 and 3, which were translocated by IGF-1 given acutely, remained elevated in the plasma membrane for 24 h. The increase in total expression of the transporters was also verified by results obtained from total lysates. In **Fig 5**(***B***) it can be seen that the total expression of GLUTs 2 and 3 was increased by insulin, whereas expression of GLUTs 1 and 5 was increased by IGF-1. Thus, chronic treatment with either insulin or IGF-1 resulted in retention of acutely translocated GLUTs in the plasma membrane with an additional increase in membrane expression of all transporters.

DISCUSSION

Glucose represents a major fuel for most mammalian cells, and a wide range of factors regulate its utilization. Indeed, impaired

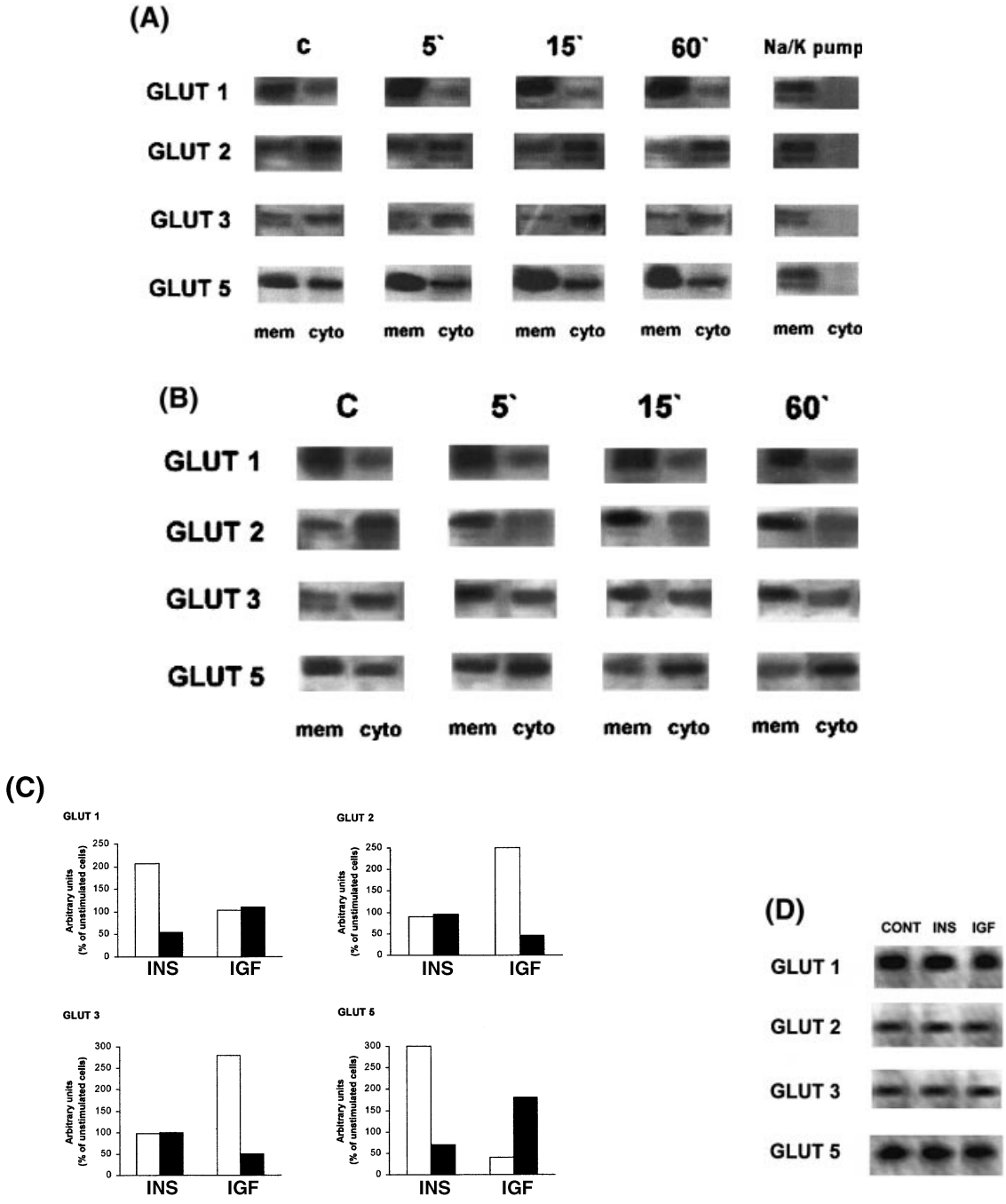

Figure 4. Acute effects of insulin and IGF-1 on the distribution of GLUTs in proliferating keratinocytes. Five-day-old keratinocytes were maintained in 0.05 mM Ca^{2+} and stimulated with 10^{-7} M insulin (*A*) or 10^{-8} M IGF-1 (*B*) for the designated times. Cells were cultivated as described in **Fig 1**. Following stimulation with insulin or IGF-1 for the designated times, cytoplasmic (cyto) and plasma membrane (mem) extracts were prepared according to *Materials and Methods*. Twenty micrograms protein were analyzed by western blotting using specific polyclonal antibodies to each transporter. The quality of the fractionation procedure was confirmed by following the expression of the Na/K pump α1 isoform, a plasma membrane protein. (*C*) Densitometry analyses of membrane (*open bars*) and cytoplasmic (*filled bars*) extracts from a representative western blot obtained in one of three separate experiments on the effects of insulin (INS) and IGF-1 (IGF) at the 60 min time period are calculated as a percentage of values obtained from control unstimulated cells. (*D*) Total expression of GLUTs in whole cell lysates was analyzed in keratinocytes stimulated for 60 min by insulin and IGF-1. Twenty micrograms protein were analyzed by western blotting using specific polyclonal antibodies to each transporter.

regulation of glucose transport and metabolism, as seen in diabetes, leads to the development of chronic complications. All tissues utilize glucose and have basal glucose transport mechanisms. Insulin-responsive tissues, such as skeletal muscle and fat, respond to acute insulin stimulation by further increasing the rate of glucose transport. Skin is not considered to be an insulin-responsive tissue, however, and its glucose transport system has not been thoroughly characterized. In this study, we have shown that proliferating skin keratinocytes indeed possess an intact glucose transport system and express four GLUT isoforms – GLUTs 1, 2, 3, and 5. In earlier studies (Gherzi *et al*, 1992; Voldstedlund and Dabelsteen, 1997), it was reported that cultured human keratinocytes apparently express GLUT 1. Similarly, GLUT 1 was identified in intact skin by immunostaining techniques (Voldstedlund and Dabelsteen, 1997).

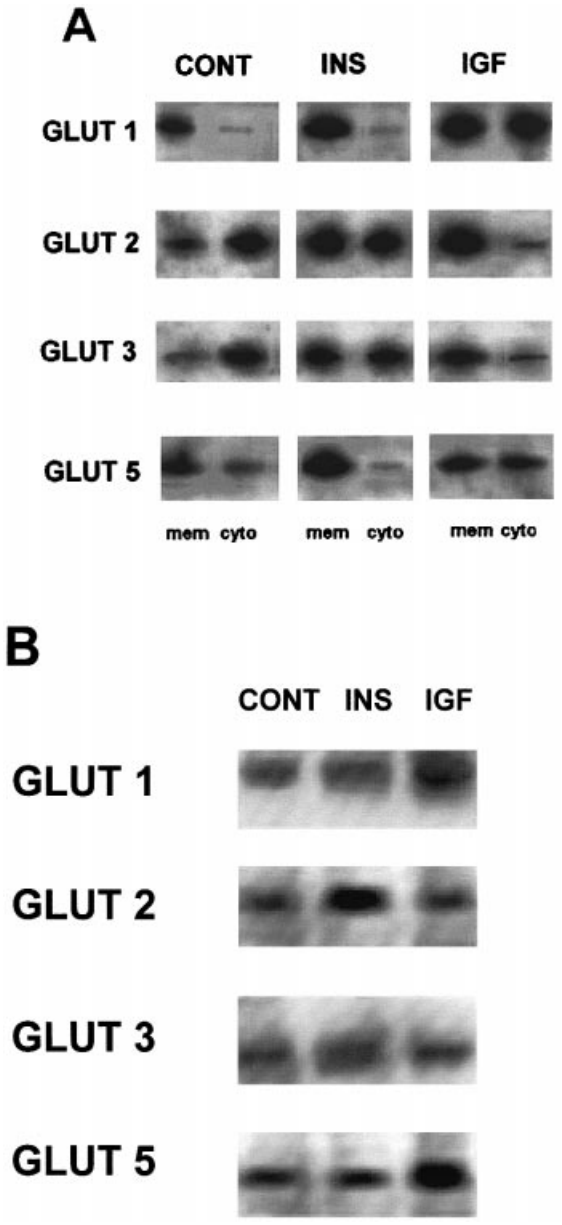

Figure 5. Chronic effects of insulin and IGF-1 on GLUT expression in proliferating keratinocytes. Western blots showing chronic effects of insulin (INS) or IGF-1 (IGF) on GLUT expression and distribution in primary keratinocytes. Cells were cultivated as described in **Fig 1**. Keratinocytes were stimulated with 10^{-7} M insulin or 10^{-8} M IGF-1 for 24 h. (*A*) Cytoplasmic (cyto) and plasma membrane (mem) extracts were prepared according to *Materials and Methods*. Twenty μg protein were analyzed by western blotting using specific polyclonal antibodies to each transporter. (*B*) Twenty micrograms of protein of total cell lysates were analyzed for GLUT expression by western blotting using specific polyclonal antibodies to each transporter.

In these studies, however, the expression of other GLUTs was not examined. Reports on a variety of tissues have shown that many cells concurrently express several GLUT isoforms. For example, GLUTs 1, 2, and 5 have been identified in other epithelial cell types, including human eye retina, corneal cells, and absorptive cells in the small intestine (Kumagai *et al*, 1994; Watanabe *et al*, 1994; Takahashi *et al*, 1996; Takata, 1996; Voldstedlund and Dabelsteen, 1997).

Interestingly, we have found that the rate of glucose transport as well as the expression levels of certain GLUTs change as a function of differentiation. Proliferating keratinocytes had the highest glucose transport rate. This was associated with a high expression level of GLUTs 1, 2, and 5. As proliferating keratinocytes have a high metabolic rate compared with the other differentiating or terminally differentiated keratinocytes, association of high glucose transport and high levels of GLUT expression with this state is obvious. As expected, induction of differentiation was associated with a downregulation in transport rate. Whereas induction of differentiation was associated with a decrease in expression of GLUTs 1, 2, and 5, however, GLUT 3 expression increased. Similar to our findings, it has been shown that expression of GLUT 3 is associated with differentiation in other tissues as well. For example, a lack of GLUT 3 was associated with abnormal muscle differentiation (Broydell *et al*, 1997; Mazzuca and Lo, 1998). Another possible explanation for the differential regulation of the various GLUT isoforms is that they may be involved in different cellular functions. Support of this notion is provided by studies showing specific intracellular localization of certain GLUT isoforms in epithelial cells. For example, in polar epithelial cells (kidney or intestine) GLUTs 1 and 2 have been shown to be expressed on the basolateral membrane, whereas GLUTs 3 and 5 are expressed on the apical membrane (Harris *et al*, 1992; Miller *et al*, 1992; Pascoe *et al*, 1996).

GLUT isoform expression was also found to be differentially regulated by hormonal stimulation. We have shown that insulin and IGF-1 stimulate glucose uptake in proliferating keratinocytes, the latter growth factor having a stronger effect than the former. Whereas insulin acutely translocated GLUTs 1 and 5, however, this change was not associated with a change in glucose transport. Moreover, although translocation from the cytoplasmic to the membrane fraction of GLUT 1 by insulin and GLUT 2 by IGF-1 was complete, only partial translocation of GLUT 5 by insulin and GLUT 3 by IGF-1 was evident. On calculating the total expression levels of the proteins in both fractions, cytoplasmic expression levels were still higher than the total expression levels in the membrane fraction. In contrast, chronic insulin as well as both acute and chronic IGF-1 treatment led to an increased expression of GLUTs 2 and 3 in the plasma membrane and this increase was associated with elevated glucose transport. These results show a discrepancy between GLUT translocation and glucose transport. Furthermore, there was an association between membrane expression of GLUTs 2 and 3 and changes in glucose transport. GLUT 1 and 5 translocation did not result in increased 2-deoxy-[^{3}H]-glucose uptake, however. Indeed it has recently been suggested that glucose transport requires not only GLUT translocation but GLUT activation as well (Sweeney *et al*, 1999). It is possible that acute insulin stimulation of proliferating keratinocytes, although leading to translocation of GLUTs, did not result in their activation.

Finally, we have found that the effects of insulin and IGF-1 were exerted exclusively on proliferating cells. There is some uncertainty, however, as to whether insulin effects are exerted via activation of insulin receptor (IR) or by activation of IGFR. Based on reports of small amounts of IR in primary cultures of human keratinocytes (Verrando and Ortonne, 1985), it was proposed that both growth factors exert their effects primarily on the IGFR (Gherzi *et al*, 1992). Although Gherzi *et al*'s findings were quantitatively consistent with this hypothesis, our findings of differential effects of insulin and IGF-1, both acutely and chronically, on GLUT translocation suggest that each growth factor acts primarily via its own receptor and signaling pathway. Moreover, functional IR and IGFR have both been shown to be expressed in primary keratinocytes (Wertheimer *et al*, 2000). Their activity is decreased during induction of keratinocyte differentiation, however (Wertheimer *et al*, 2000). Furthermore, in several cell models including cardiac myocytes and adipocytes, insulin-induced glucose uptake was found to be also mediated by the GLUT 1 transporter in addition to GLUT 4. Thus, the actual decrease in expression levels of the various GLUTs during keratinocyte differentiation could explain the lack of responsiveness to insulin stimulation (Calderhead *et al*, 1990; Robinson and James, 1992; Fischer *et al*, 1997; Egert *et al*, 1999). Altogether, these results might explain the lack of response of the glucose transport system

to insulin and IGF-1 stimulation in differentiating cells. Nonetheless, insulin and IGF-1 may exert other as yet undescribed metabolic effects on differentiating keratinocytes. Studies are currently being undertaken to clarify the major signaling mechanisms in insulin- and IGF-1 induced effects in skin cells.

In summary, our results demonstrate that skin keratinocytes possess a hormone-sensitive glucose transport system that is regulated during differentiation. Furthermore, we have shown that insulin and IGF-1 differentially regulate the glucose transport system. Further studies are required to identify the specific role of insulin, IGF-1, and the glucose transport system in normal as well as pathologic skin physiology.

Supported in part by the Sorrell Foundation and a grant from the Israel Science Foundation funded by the Israel Academy of Sciences. This work was supported by a grant from the Smokeless Tobacco Research Council. EW is a recipient of a Career Development Award from the Juvenile Diabetes Foundation International. SRS is the incumbent of the Louis Fisher Chair in Cellular Pathology.

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